Immunopotentiating Effect of a *Fomitella fraxinea*-Derived Lectin on Chicken Immunity and Resistance to Coccidiosis

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ABSTRACT This study reports a novel immunopotentiating effect of a lectin (FFrL) extracted from the mushroom *Fomitella fraxinea* on poultry cell-mediated immunity and poultry coccidiosis. We describe the extraction of FFrL, its in vitro mitogenic activity and in vivo protection against an oral challenge infection with *Eimeria acervulina*. When tested on several cell types, crude FFrL agglutinated mouse erythrocytes and thymocytes and various other cells including murine and human cell lines. However, crude FFrL did not agglutinate human erythrocytes. Crude FFrL showed a potent mitogenic activity on chicken splenic lymphocytes, and at lower concentrations it exerted stronger mitogenic activity than Concanavalin A, a well-known potent mitogen for lymphocytes. Further, FFrL significantly induced ($P < 0.05$) nitric oxide secretion in HD11 cells and suppressed ($P < 0.05$) RP9 tumor cell growth in a dose-dependent fashion. When injected into 18-d-old chicken embryos followed by a posthatch oral *E. acervulina* challenge infection, FFrL treatment significantly protected chickens against weight loss associated with coccidiosis ($P < 0.05$). Injecting embryos with FFrL also resulted in significant reduction in oocyst shedding as compared with the control saline-injected birds ($P < 0.05$). The results of this study demonstrate that FFrL can be an effective growth promoting and immunostimulating agent in poultry during coccidiosis.

Key words: coccidiosis, lectin, *Fomitella fraxinea*, in ovo, cell-mediated immunity

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INTRODUCTION

Mushrooms and mushroom lectins have recently gained significant attention in medical research because of their immunoenhancing effects and their demonstrated potential in promoting health (Borchers et al., 2004). Lectins are carbohydrate-binding proteins or glycoproteins of nonimmune origins with the ability to induce cell agglutination (Goldstein et al., 1980). They are found in diverse living organisms including animals, plants, and microorganisms (Cammue et al., 1985; Suzuki, 1985; Avichezer and Gilboa-Garber, 1991). Lately, there has been a growing interest in lectins, largely due to the discovery that some induce various important biological activities including immunomodulatory activities (Wang et al., 1996 and 2002; She et al., 1998; Lima et al., 1999; Ho et al., 2004), antiproliferative/antitumor activities (Wang et al., 1996; Abdullaev and de Mejia, 1997; Yoon et al., 1999; Zhao et al., 2003; Ngai and Ng, 2004), antifungal activities (Ye et al., 2001) and antiviral activities (Marchetti et al., 1995; Ye et al., 2001). Although many mushroom lectins have been isolated and characterized (Guillot and Konska, 1997), only some of them have been shown to possess immunomodulatory activity. More recently, some mushroom extracts were shown to have immunoenhancing potential in chickens (Guo et al., 2004, 2005), particularly during coccidiosis.

Avian coccidiosis is the major parasitic disease of poultry with substantial economic burden to the industry. Infeed medication for prevention and treatment contributes a major portion of economic costs, and associated losses are also due to mortality, malabsorption, inefficient feed use and impaired growth rate in broilers, and a temporary reduction of egg production in layers. Coccidiosis is caused by several apicomplexan parasites of the genus *Eimeria* that infect the intestinal tract and are transmitted between birds via ingestion of infective oocysts. Although natural infection and live oocyst vaccination with *Eimeria* spp. induce immunity, disease control remains largely dependent on routine use of anticoccidial drugs (Lillehoj et al., 2004; Dalloul and Lillehoj, 2005). However, the demand for more efficient vaccines, the increasing incidence of drug-resistant strains, the escalating public anxiety over chemical residues in meat and eggs, and the regulatory bans of growth-promoting drugs in poultry production mandate the development of alternative control methods. In this study, we investigated the immuno-

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potentiating effect of a mushroom lectin (**FFrL**) extracted from *Fomitella fraxinea* on poultry cell-mediated immunity and subsequent protection against coccidiosis.

MATERIALS AND METHODS

Preparation of the Crude FFrL

The carpophores of *F. fraxinea* were collected at Cheonggye Mountain in Gyung-Ki Province, South Korea, and kept frozen at –70°C until use. Two kilograms of the frozen fruiting bodies were homogenized in 20 L of 20 m*M* Tris-HCl buffer (pH 8.0) with a blender and then extracted for 18 h at 4°C with frequent gentle swirling. The resulting suspension was filtered, and ammonium sulfate was added to the filtrate to 50% saturation. After standing overnight at 4°C, the resulting precipitate was separated by centrifugation at 8,000 × *g* and 4°C for 20 min. The supernatant was collected, and more ammonium sulfate was added to the supernatant to obtain 100% saturation. After standing overnight at 4°C, the resulting precipitate (from the second ammonium sulfate precipitation) was separated by centrifugation as just described. The precipitate was dissolved in 50 m*M* NaCl, 50 m*M* Tris-HCl buffer (pH 8.0), dialyzed against distilled water for 5 to 7 d at 4°C with frequent changing of distilled water. After dialysis, the dialysate was freeze-dried to yield crude FFrL, a grayish brown amorphous powder freely soluble in distilled water as well as saline and tissue culture media (e.g., RPMI 1640) but not soluble in organic solvents such as ethanol, acetone, chloroform, or diethyl ether. Except for the hemagglutinating activities, only the crude fraction of FFrL is reported here.

Downstream purification yields more active FFrL as verified by its ability to agglutinate erythrocytes of BALB/c mice. The FFrL was further purified using different HPLC methods (2 different ion-exchange column chromatography and 1 gel filtration). The lyophilized extract (i.e., crude FFrL) was dissolved in 20 m*M* Tris-HCl buffer (pH 8.0) and applied on a DEAE-cellulose column (2.5 × 20 cm; Sigma, St. Louis, MO) and eluted with the same buffer. Each fraction was assessed for hemagglutinating activity as described later. The hemagglutinating fractions were pooled, dialyzed against 20 m*M* Tris-HCl buffer (pH 8.0), and then loaded onto a DEAE-sephacel column equilibrated with the same buffer. The bound components were eluted with a linear gradient of 0 to 0.4 mol/L NaCl in 20 m*M* Tris-HCl buffer (pH 8.0). The active fractions were pooled and further purified with gel filtration on a Sephacryl S-200 HR column (Sigma). The purified lectin fractions were pooled, dialyzed against distilled water, and then lyophilized, giving a purified lectin FFrL.

Hemagglutination Test

In the assay for hemagglutinating activity, a serial 2-fold dilution of the lectin solution in microtiter U-plates (20 μL) was mixed with 30 μL of a 2% suspension of BALB/c mouse red blood cells in PBS (pH 7.2) at 37°C. The results were read after 30 min. The hemagglutinating titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was reckoned as hemagglutinating activity unit (U).

Mitogenic Activities on Chicken Splenic Lymphocytes

The mitogenic activity of FFrL on chicken splenic lymphocytes was measured by the nonradioactive, enzymatic XTT assay (Roehm et al., 1991). Spleen cells were prepared from 3 freshly harvested chicken spleens as described by Dalloul et al. (2002) and cocultured in triplicate wells with varying concentrations (0, 1.56, 3.13, 6.25, 12.5, and 25.0 μg/mL) of either FFrL or concanavalin A (**ConA**) as positive control. Cells were cultured in a 5% CO_2 atmosphere at 41°C for 48 h, and the cell growth was then assessed by the XTT colorimetric method following either a 3- or a 5-h reaction and expressed as optical density read at 450 nm.

Induction of Nitric Oxide Production in Macrophages

Induction of nitric oxide production was assayed using the chicken macrophage cell line HD11 stimulated by FFrL and controls. Cells were cultured in 96-well plates at a concentration of 1×10^5/well (100 μL) and stimulated with 100 μL of RPMI-complete medium (negative control), recombinant chicken interferon-gamma expressed in COS-7 cells (IFN-γ; positive control), or varying concentrations of FFrL (12.5, 25.0, 50.0, or 100.0 μg/mL) all in triplicates. Cells were cultured in a 5% CO_2 atmosphere at 41°C for 24 h, and nitric oxide was assessed in triplicate wells as nitrite content in conditioned media using Griess reagent as described (Ding et al., 1988). Mean nitrite values (μM) were calculated using a sodium nitrite standard curve.

Suppression of Tumor Cells

To test the antitumor activity of FFrL we used the LSCC-RP9 B lymphoblastoid cell line, which is derived from tumor induced by Rous-associated virus 2 and is commonly used as chicken target cell line. The RP9 cells (5×10^4 in 100 μL/well of 96-well plates) were cultured with 100 μL of RPMI-complete medium as negative control, human TNF-α as positive control (3 μg/mL; R&D System, Minneapolis, MN), or increasing concentration of FFrL (12.5, 25.0, 50.0, or 100.0 μg/mL) all in triplicates. Cultures were incubated in a 5% CO_2 atmosphere at 41°C for 24 h, and cell viability was assessed in triplicate wells using the WST-8 tetrazolium salt assay (Cell-Counting Kit-8, Dojindo Molecular Technologies, Inc., Gaithersburg, MD) as described by Miyamoto et al. (2002). The results were expressed as optical density read at 450 nm.

Table 1. Purification and hemagglutinating activity (HA) of the *Fomitella fraxinea*-derived lectin (FFrL)[1]

Purification step	Total protein (mg)	Specific activity[2] (HA U/mg)	Total activity[3] (U)	Fold purification	Recovery (%)
Crude FFrL	3,039.0	64	194,496	1	100.0
DEAE-cellulose	279.5	256	71,552	4	36.8
DEAE-Sephacel	171.1	256	43,801	8	22.5
S200-HR	10.5	4,096	43,008	64	22.1

[1]Upon purification, FFrL activity was verified by its ability to agglutinate BALB/c erythrocytes (triplicates) as described in the text. The purification steps resulted in a 64-fold purification with a 22.1% yield.

[2]Specific activity (HA units): calculated as the inverse of the minimum concentration producing a positive reaction in the hemagglutination assay when using 1 mg of FFrL.

[3]Total HA activity: calculated as specific activity (HA units) of total starting material in milligrams of each FFrL fraction.

Protective Effect of FFrL Against Poultry Coccidiosis

Experimental Birds and In Ovo Injection. Eighteen-day-old specific-pathogen-free embryonated chicken eggs (White Leghorn SPAFAS, Charles River Laboratories, Storrs, CT) were injected into the amniotic cavity with either PBS (30 eggs; 100 μL per egg) as control or FFrL extract (15 eggs; 100 μg in 100 μL per egg) using a customized Intelliject in ovo injection system (AviTech LLC, Hebron, MD). Birds were hatched at the Animal and Natural Resources Institute (USDA, Beltsville, MD), housed in heated brooding units, wing-tagged, and feed and water were provided ad libitum throughout the experimental period. All animal protocols followed the guidelines of the Institutional Animal Care and Use Committee of the USDA Beltsville Agricultural Research Center.

Eimeria Infection and Assessment of Fecal Oocyst Production. One week posthatch, each bird (except the negative control groups) received an oral dose of 10,000 sporulated *Eimeria acervulina* (**EA**) oocysts and transferred into small cages at 2 birds/cage. Fecal materials were collected 6 to 9 d postinfection, processed, and the number of shed oocysts counted. Oocyst production and shedding were assessed as described by Dalloul et al. (2005). Briefly, collected fecal samples were soaked overnight, ground, and homogenized. Two 35-mL samples were taken, diluted, and the oocysts counted microscopically using a McMaster counting chamber (HK Inc., Tokyo, Japan). The number of oocysts per bird was calculated using the formula: total number oocysts = oocyst count × dilution factor × (fecal sample volume/counting chamber volume)/number of birds per cage. All birds were individually weighed at 0 and 9 d postinfection.

Statistical Analysis

Differences between experimental treatments were tested by 1-way ANOVA (InStat, GraphPad Software Inc., San Diego, CA) and were considered significant at $P < 0.05$ by the Tukey-Kramer Multiple Comparisons Test.

RESULTS

Hemagglutinating Activities of Crude FFrL

The FFrL activity was checked by its ability to agglutinate BALB/c erythrocytes (Table 1). Downstream purification of FFrL using different HPLC methods resulted in a 64-fold purification with a 22.1% yield; however, only crude FFrL is reported in this study. Crude FFrL agglutinated not only the erythrocytes of BALB/c mice, but also various other cells including thymocytes of BALB/c mice, erythrocytes of Sprague-Dawley rats, mouse RAW 264.7 cells, mouse sarcoma 180 cells, human THP-1 cells, and human cervical carcinoma HeLa cells (unpublished data). However, crude FFrL did not agglutinate human A, B, AB, or O erythrocytes.

Mitogenic and Antitumor Activities

The mitogenic activity of FFrL on the splenic lymphocytes of chickens was measured by the XTT assay. As depicted in Figure 1, FFrL showed a potent mitogenic activity especially at lower concentrations, where crude

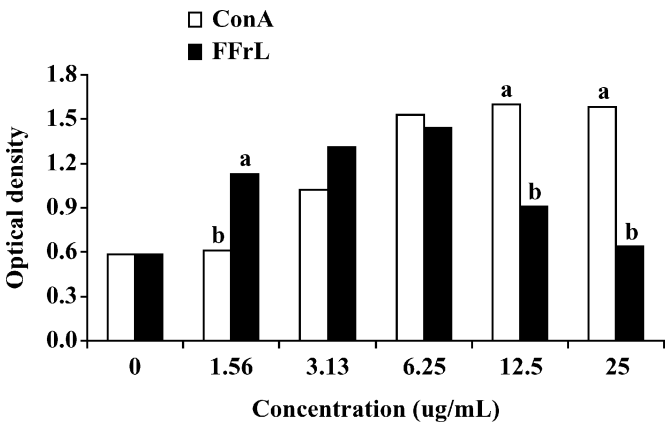

Figure 1. Mitogenic activity of crude *Fomitella fraxinea*-derived lectin (FFrL) on chicken splenic lymphocytes. Spleen lymphocytes were prepared from 3 freshly harvested chicken spleens and cocultured in triplicate wells with the stimulants in 5% CO_2 atmosphere at 42°C for 48 h, and then the cell growth was assessed by the XTT colorimetric method (optical density was read at 450 nm). ConA = concanavalin A. $^{a–b}P < 0.05$.

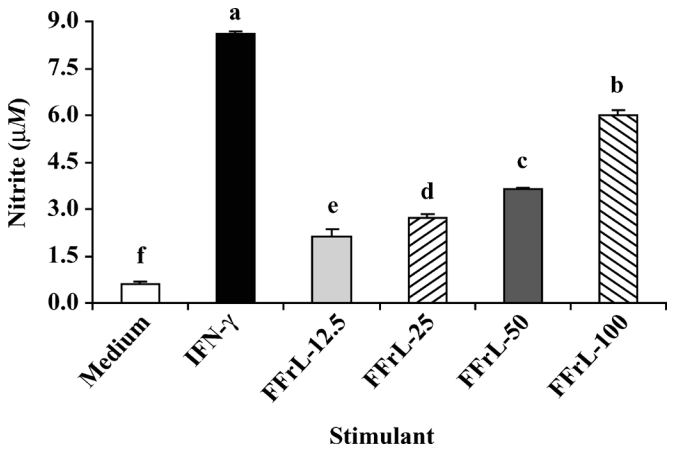

Figure 2. Nitric oxide secretion (μ*M*) by HD11 cells 24 h following IFN-γ or *Fomitella fraxinea*-derived lectin (FFrL) stimulation. Cells were cultured in triplicates in 96-well plates at a concentration of 1×10^5/well (100 μL) and an equal volume of appropriate controls and FFrL added at multiple concentrations (μg/mL). Supernatants (100 μL) of activated cells were transferred to new 96-well plates (triplicates), 100 μL of Griess reagent was added, and the optical density was read at 540 nm. $^{a–f}P < 0.05$; error bars = SE.

FFrL exerted stronger mitogenic activity than ConA, a well-known potent mitogen. However, the activity declined with higher concentrations just contrary to that of ConA. Further, FFrL significantly induced ($P < 0.05$) nitric oxide secretion in HD11 cells (Figure 2) and suppressed ($P < 0.05$) RP9 cell growth (Figure 3), both in a dose-dependent fashion. The RP9 tumor cell growth was even more suppressed by the highest FFrL concentration than human TNF-α (Figure 3).

Protective Effect of FFrL Against Poultry Coccidiosis

Eighteen-day-old chicken embryos were injected with either PBS (100 μL per egg) as control or FFrL extract

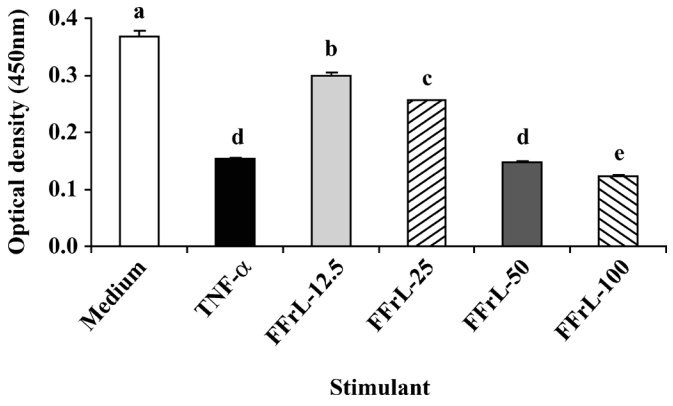

Figure 3. The effect of *Fomitella fraxinea*-derived lectin (FFrL) on the viability of RP9 cells was measured by the WST-8 tetrazolium salt assay. Cells were cultured in 96-well plates at a concentration of 5×10^4/well (100 μL; triplicates) and an equal volume of appropriate controls and FFrL added at multiple concentrations (μg/mL). After 24 h of incubation, 10 μL of CCK-8 reagent was added to each well. Plates were further incubated for 4 h at similar conditions, and the optical density was read at 450 nm. $^{a–e}P < 0.05$; error bars = SE.

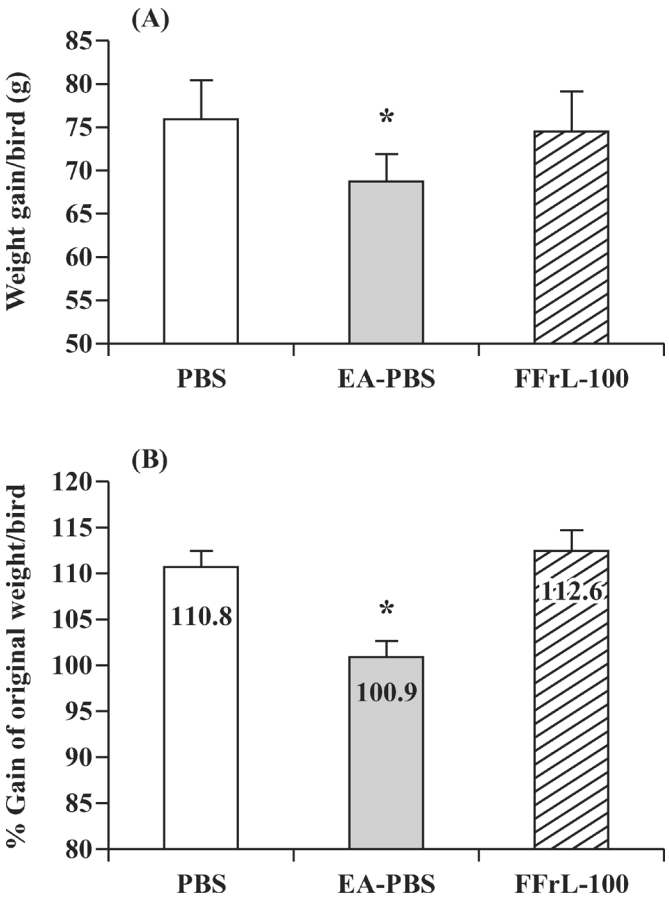

Figure 4. Body weight gains during *Eimeria acervulina* (EA) infection. Embryos (18 d old) were injected into the amniotic cavity with PBS (n = 30; 100 μL/egg) or crude *Fomitella fraxinea*-derived lectin (FFrL; n = 15; 100 μg/100 μL), and hatched chicks (n = 12 to 15/group) except for noninfected PBS controls were inoculated with 10,000 *E. acervulina* oocysts at 6 d of age. Birds were individually weighed on 1 and 9 d postinfection (dpi), and both weight gain (A) and percentage gain (B) were statistically analyzed by ANOVA and the Tukey-Kramer post hoc test. *Indicates significantly ($P < 0.05$) different than the noninfected controls (PBS); error bars = SE.

(100 μg/egg), and hatched birds were orally infected with *E. acervulina* oocysts. Body weight gains and fecal oocyst shedding were evaluated during the infection period. The FFrL-treated and EA-infected chickens showed significantly higher weight gains ($P < 0.05$) than infected controls (Figure 4A), and similar weight gains to those of normal noninfected chickens. The percentage of BW gain per bird was also computed and 1-way ANOVA applied as described earlier (Figure 4B). Percentage of BW gain was significantly lower ($P = 0.003$) in the infected control group (EA-PBS) as compared with either the noninfected (PBS) or the FFrL-injected, infected (FFrL) groups. No differences ($P > 0.05$) were found between the noninfected (PBS) and the FFrL-injected (FFrL-100) groups. Furthermore, FFrL injection of embryos also resulted in significantly lower fecal oocyst shedding as compared with PBS-injected birds following *E. acervulina* challenge (Figure 5).

DISCUSSION

To prevent and control coccidiosis, the poultry industry has relied heavily upon prophylactic chemotherapy re-

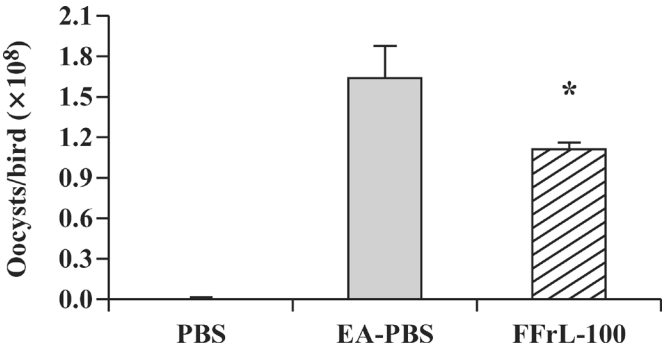

Figure 5. Oocyst shedding by chicks 6 to 9 d following *Eimeria acervulina* (EA) infection. Embryos (18 d old) were injected into the amniotic cavity with either PBS (n = 30; 100 μL) or crude *Fomitella fraxinea*-derived lectin (FFrL; n = 15; 100 μg/100 μL), and hatched chicks (n = 12 to 15/group) were inoculated with 10,000 *E. acervulina* oocysts at 1 wk of age. Fecal materials (2 birds/cage) were collected 6 through 9 d postinfection (dpi), processed, and shed oocysts counted. *Indicates significantly ($P < 0.05$) different than the infected controls (EA-PBS); error bars = SE.

sulting in the development of resistant strains of *Eimeria* to all introduced anticoccidial drugs (Chapman, 1997). Therefore, recent research has focused on the development of alternative disease control strategies including the introduction of alternative prevention/treatment measures such as nonchemical feed supplements (Dalloul et al., 2003a,b), and novel and effective vaccines like recombinant (Ding et al., 2004; 2005; Lillehoj et al., 2005) and new live (Weber et al., 2004) vaccines, and other immunization strategies such as the use of CpG oligodeoxynucleotides (Dalloul et al., 2004, 2005) and mushroom and herb extracts (Guo et al., 2004, 2005). We therefore tested the potential effects of a mushroom-derived lectin in inducing immunoprotection against an *Eimeria* challenge. *Fomitella fraxinea* is a wood-rotting basidiomycetous fungus growing mostly on the tree stumps of *Robinia pseudoacacia*. It is a very common wild mushroom widely distributed in Korea as well as other countries including the United States.

Very limited research exists on the effects of mushroom and their extracts on poultry health particularly during coccidiosis. Recent work with other mushrooms and mushroom-extracted polysaccharides has shown in vivo protective effects against *E. tenella* infection (Guo et al., 2004, 2005). These mushrooms and their polysaccharide extracts showed promise in altering bacterial activities and composition in chicken ceca. The polysaccharide extracts showed a slightly significant effect on growth performance but had no effects on weights of immune and gastrointestinal tract organs in normal broilers. However, when used in conjunction with a vaccine, those extracts showed significant effects on body growth, immune responses as well as growth of immune organs and development of gastrointestinal tract fragments in coccidian-infected chickens. The authors concluded that supplementation of mushroom or herb extracts, or both, in the poultry diet resulted in enhancement of resistance to *E. tenella* probably by enhancing both cellular and humoral immune responses against *E. tenella* in chickens. Indeed, the improved resistance to coccidiosis shown in this work could be due to nonspecific as well as specific immunoenhancement exerted by the injected mushroom lectin that improved innate and adaptive immune responses.

Even though similar results were observed in the current study, the mushroom extract used is a lectin that is usually prepared under much less stringent conditions, making it more feasible to be produced commercially. Further, the lectin was coupled with successful in ovo delivery, offering a promising means of controlling coccidiosis. In conclusion, growth performance of *E. acervulina*-infected chickens was significantly improved by injecting the FFrL into 18-d-old embryos as best manifested by higher weight gains over the infected control birds. The FFrL-treated chickens also showed significantly reduced oocyst shedding after oral challenge infection with live parasites, an indication of improved resistance to coccidiosis. In view of increasing evidence that mushroom and mushroom-derived lectin enhance innate immunity in poultry, better characterization of the mechanism of their action at cellular and molecular levels will be necessary before they can be used as immunopotentiators in poultry and other livestock.

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